IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Gerianne Tringali DiPiano, Peter Kevin Mays and John Ziemniak		
Serial No.:	10/751,056	Group Art Unit:	1617
Filed:	January 2, 2004	Examiner:	Jennifer M. Kim
For:	*PHARMACEUTICAL PREPARATIONS FOR TREATMENTS OF DISEASES AND DISORDERS OF THE BREAST*		

Commissioner for Patents
P.O. Box 1450
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DECLARATION UNDER 37 C.F.R. § 1.132

The undersigned, Peter Kevin Mays, Ph.D., do hereby declare and state that:

1. I am a co-inventor of the above-identified application.

2. I am currently the Vice President of Pharmaceutical Development, at FemmePharma Global Healthcare, located in Wayne, Pennsylvania. I manage all pharmaceutical development activities including drug formulation and supplying clinical trial material.

3. I have worked in the regulated medical device and pharmaceutical industry for 17 years developing drugs and devices for the U.S. marketplace. Prior to joining industry I completed a Ph.D. in biochemistry and post-doctoral training in molecular biology.

4. I have reviewed the Office Action mailed May 1, 2009, in connection with the above-identified application and the following references applied by the Examiner in that Office Action:

a. U.S. Patent No. 5,993,856, Ravagan, *et al.* ("Ravagan")

b. U.S. Patent No. 5,614,212, D'Angelo, *et al.* ("D'Angelo")

5. I understand that claims 1-5, 7, and 8 are rejected as being obvious in view of Ravagan, which discloses a transvaginal formulation of danazol nano or microparticulates. The following provides evidence demonstrating that a transvaginal danazol formulation is ineffective when applied to the skin, because it does not permeate the stratum corneum. By comparison, the combination of a hydroalcoholic gel excipient and a penetration enhancer results in permeation of danazol across the stratum corneum.

6. The work described in the enclosed exhibits was carried out by Absorption Systems, in Exton, PA, on behalf of FemmePharma, Inc.

7. A topical, transvaginal formulation containing danazol was tested for permeability across dermatomed human abdominal skin using a Franz cell-diffusion assay. The formulation of the test compound is found in Exhibit 1, entitled "1.1.1. Quantitative Composition." The compound was placed on the dry surface of the skin. Samples were taken from the fluid reservoir in contact with the opposing surface at discrete time points between 0 and 48 hours, and the presence of danazol was measured by LC-MS/MS (see Exhibit 2, entitled Confidential Final Report, Study Number 8FEMMP1).

8. The presence of danazol was not detected in the receiver chamber at any time point during the assay (see Exhibit 2, page 6, Table 3).

9. This result demonstrates that a topical, transvaginal formulation does not permeate the skin.

10. A study was carried out to determine the effect of an alcoholic excipient on the permeation of danazol across the stratum corneum (Exhibit 3, entitled Study Report No.:

02-FEMM.P01.R1). Two compounds, a Propylene Glycol formulation containing 10 ml propylene glycol, 25.63 mg lucifer yellow, 100.64 mg danazol; and a 5% Oleyl Alcohol in PG formulation containing 9.5 ml propylene glycol, 0.5 ml oleyl alcohol, 25.63 mg lucifer yellow, 100.64 mg danazol were compared for their ability to permeate human breast skin using a Franz-cell diffusion assay. Each compound was placed on the dry surface of the skin. Samples were taken from the fluid reservoir in contact with the opposing surface at discrete time points between 0 and 48 hours to measure the presence of danazol by liquid chromatography/mass spectroscopy. Following the assay, the percentage of danzol remaining in the donor compartment was also determined.

11. Permeability of danazol through the skin was clearly enhanced in the presence of oleyl alcohol, as demonstrated by an increasing accumulation of danazol in the receiver compartment over time between 8 and 48 hours (see Exhibit 3, page 4, Figure 1).

12. Residual compound from the donor chambers was collected at the end of the 48 hour incubation period and assayed for the presence of danazol. High percentages of danazol were recovered from the donor compartment of the propylene glycol formulation, compared to low percentages for the formulation containing oleyl alcohol (see Exhibit 3, page 5, Table 3). These results are consistent with an increase in permeation of danazol into and/or through the skin in the presence of an alcohol excipient.

13. The test material of Paragraph 10, containing oleyl alcohol, danazol, lucifer yellow and propylene glycol, demonstrated that danazol could be delivered across a stratum corneum. However, the rate of flux was low (0.055 $\mu g/cm^2/hr$) (Exhibit 3, page 5, Table 2).

14. To improve the rate of flux, we conducted formulation trials in which we used 2-pyrrolidone (synonym - 2-pyrrolidinone), a known permeation enhancer, in various formulation bases (see Exhibit 4, entitled "Formulation Development Summary on Danazol."). Some of these were tested for their ability to improve danazol flux across the stratum corneum.

15. Hydro-alcoholic gel #1, Hydro-alcoholic gel #2, and PEG ointment #1 (described in Exhibit 4, pages 1-3), all containing danazol and the penetration enhancer 2-pyrrolidone, were individually applied to human breast skin in a Franz-cell diffusion assay for a 48 hour incubation period.

16. Samples were taken from the fluid at discrete time points between 0 and 48 hours to measure the presence of danazol by liquid chromatography/mass spectroscopy. Skin permeability of danazol was clearly enhanced in the presence of a hydro-alcoholic gel, as demonstrated by an increasing accumulation of danazol in the receiver compartment at time points between 8 and 48 hours of both hydro-alcoholic gel #1 and #2, compared to PEG ointment #1 (see Exhibit 5, labeled Study Report No.: 04-FEMM.P01.R2, page 4, Figure 1).

17. Residual compound from the donor chambers was collected at the end of the 48 hour incubation period and assayed for the presence of danazol. High percentages of danazol were recovered from the donor compartment of the PEG ointment #1, compared to low percentages for hydro-alcoholic formulations (see Exhibit 5, page 6, Table 4). These results are consistent with an increase in permeation of danazol into and/or through the skin in the presence of an alcohol excipient and skin penetration enhancer.

18. In the PEG ointment #1 15% pyrrolidone did not enhance danazol flux (0.004 $\mu g/cm^2/hr$), whereas in hydroalcoholic gel # 2 15% pyrrolidone in the presence of 47% alcohol enhanced the danazol flux rate (0.127 $\mu g/cm^2/hr$), a rate of flux twice that seen with oleyl alcohol alone (Exhibit 5, page 5, Table 2). This finding was unexpected.

19. The combination of Danazol + Gel + Alcohol + PVP is not relevant to this application, as PVP, a known penetration enhancer, is a polymer which confers different physicochemical properties to the formulation than 2-pyrrolidone, which is a low molecular weight small molecule. Within the formulation of hydroalcoholic gel #2, the 2-pyrrolidone acts as both a solubilizer for danazol and a penetration enhancer.

20. A human clinical study was conducted using hydroalcoholic gel #2 in 15 premenopausal women with fibrocystic breast disease. Each woman applied 2 g of hydroalcoholic gel, containing a total of 40 mg of danazol, to her breasts everyday for four months. The drug product was well tolerated, there were no systemic androgenic side-effects, serum levels of danazol at steady state were <2.75 ng/mL, and over four months breast pain was reduced by 54%. These data demonstrate that danazol may be formulated into a transdermal gel to deliver drug to treat fibrocystic breast disease with low systemic exposure to danazol.

21. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

27 August 2009
Date

Peter Kevin Mays, Ph.D.

1.1.1 Quantitative Composition

The following tables lists the quantitative composition for the stability lot and clinical supplies:

Table 7.2: Quantitative Composition

Component	Amount (mg/dose)	Percentage (%)
Danazol, USP	100.00	46.99
Carbopol 934 NF	0.33	0.16
Glycerin, USP	13.33	6.26
Methylparaben, NF	0.28	0.13
Trolamine, NF	0.5	0.23
Purified Water, USP qs	98.39	46.23
TOTAL	212.83	100

CONFIDENTIAL

FINAL REPORT

STUDY NUMBER
8FEMMP1

Unidirectional Permeability Assessment of One Formulation of the Customer's Test Compound (Danazol) Across Dermatomed Human Skin

SUMMARY
The objective of this study was to determine the uni-directional permeability of the Customer's test compound, topical formulation of danazol, across frozen dermatomed human skin tissue.

Tissue integrity marker, lucifer yellow, was below LLOQ in all receiver buffer indicates that the donor used in this assay was acceptable until 48 hours on the tissue integrity assessment, since all replicate was passed by lucifer yellow permeation. The P_{app} of low permeable control compound, atenolol, exhibited $0.02 \pm 0.04 \times 10^{-6}$ cm/s and that of high permeable control compound, caffeine, exhibited $0.10 \pm 0.12 \times 10^{-6}$ cm/s.

However, the flux of the customer's test compound, danazol, was zero in all replicate, since danazol was not detected in all receiver compartment. It was probably caused by that the customer's formulation, a solid cream type formulation, was not suitable for the Franz-cell diffusion assay utilized in this assay because of the difficulties of spreading on the skin surface to cover the entire surface tightly by the characteristics of this formulation. Another possibility is that the danazol may also have accumulated in the skin tissue and not have been released into the receiver compartment since danazol is very insoluble and lipophilic.

DATE OF ISSUE
December 4, 2008

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COMPLIANCE
This study followed established practices and standard operating procedures of Absorption Systems LP. The report is archived in a validated Scientific Data Management System. Electronic signatures comply with the regulation 21 CFR Part 11.

ABSORPTION SYSTEMS
The Drug Absorption Company

1. OBJECTIVE

The objective of this study was to determine the uni-directional permeability of the Customer's test compound, topical formulation of danazol, across frozen dermatomed human skin tissue.

2. MATERIALS AND METHODS

Materials

Lucifer yellow was purchased from Molecular Probes (Eugene, OR). Atenolol, caffeine, and Krebs-Ringer bicarbonate buffer were obtained from Sigma-Aldrich (St. Louis, MO). The Customer supplied the topical formulation of danazol and its powder. Blank Krebs-Ringer Bicarbonate buffer (KRB buffer) was modified by including 1.2 mM $CaCl_2$, and 10 mM HEPES at pH 7.4.

Tissue Preparation and Permeability Assay

Dermatomed human abdominal skin was obtained from Asterand Inc. (Detroit, MI). The donor was a 77-year-old Caucasian Female. It was kept frozen at -80°C until the time of the study. After thawing at room temperature, the skin was soaked in saline for 30 minutes, and then cut to the appropriate size (~ 4 cm^2) necessary. Excess moisture and saline were wiped off. The tissue was immediately transferred to the Franz-cell diffusion chamber and clamped between the donor and receiver chambers. The exposed surface area of the Franz-cell diffusion chamber was 1.77 cm^2. The receiver compartment was filled with 8 mL KRB buffer. The reservoir also contained a stirring bar to mix the reservoir contents. The stirring rate was set at 10 (400 RPM). Each Franz-cell diffusion chamber was then placed in a dry block heating/stirring module. The temperature was set to maintain the tissue surface at 32°C.

The Customer's formulation containing 47% of danazol (Table 1) was directly rubbed into the donor compartment on the top of the skin tissue. However, the customer's formulation did not have an appropriate softness because of the solid cream type formulation, it was difficult to spread to the skin surface to cover the entire surface, and the formulation was unable to be stacked to the surface tightly. For parallel control compound assessment, a dosing vehicle containing the tissue integrity marker, lucifer yellow (100 µM), the low permeability reference, atenolol (100 µM), and the high permeability reference, caffeine (100 µM) in KRB buffer (2 mL), was placed directly into the donor compartment on the top of the tissue surface (this treatment did not involve any formulation).

Table 1. Weight of Test Compound Formulation

	Chamber 1	Chamber 2	Chamber 3	Chamber 4	Mean ± S.D.
Formulation 1	0.6846 g	0.5811 g	0.6929 g	0.6733 g	0.6580 ± 0.0519 g

Samples (1.0 mL) were taken from the receiver compartment at 0, 2, 4, 8, 24, and 48 hours for formulation assays and the control assay. After taking samples, an equal volume of receiver KRB buffer was added back to replace the buffer removed. For the control assay, 200 µL of dosing solution (prior to 0 hours) was taken from the container and 200 µL of 0 and 48 hours samples were taken from the donor side of the chamber.

Sample Analysis

Lucifer yellow concentrations in the control assay were measured using a FLUOstar fluorescence plate reader (BMG Laboratories, Durham, NC). The excitation and emission wavelengths were 485 and 538 nm, respectively.

The test compound, danazol, and control compounds, atenolol and caffeine, were measured by LC-MS/MS, and the method is described in Appendix I.

Data Analysis

For the test compound, danazol, the fluxes (nmole/cm^2/hour) was calculated, because of no donor analysis, from a linear part of the slope of the cumulative amount permeating into the receiver side through a unit of surface area of the skin (nmole/cm^2) as a function of time. The following calculations were performed:

$$\text{Flux} = d[(C_r \times V_r) / A] / dt$$

where,
C_r is the cumulative receiver compartment concentration in nM
V_r is the volume of the receiver compartment, 8.0 mL
A is the diffusional surface area of the exposed skin membrane, 1.77 cm^2

For the QC compounds, lucifer yellow, atenolol, and caffeine, the apparent permeability coefficient (P_{app}) and percent recovery were calculated as follows:

$$P_{app} = (dC_r/dt) \times V_r / (A \times C_0)$$

$$\text{Percent Recovery} = 100 \times ((V_r \times C_r^{final}) + (V_d \times C_d^{final})) / (V_d \times C_0)$$

Where,
dC_r/dt is the slope of the cumulative receiver compartment concentration versus time, $\mu M \cdot min^{-1}$.
A is the diffusional surface area of the exposed skin membrane, 1.77 cm^2
V_r is the volume of the receiver compartment, 8.0 mL
V_d is the volume of the donor compartment, 2.0 mL.
C_r is the cumulative receiver compartment concentration in µM
C_0 is the concentration of the donor at 0 minutes of the incubation, µM.
C_r^{final} is the concentration of the receiver at the end of the incubation period, µM.
C_d^{final} is the concentration of the donor at the end of the incubation period, µM.

3. RESULTS

The raw data of donor and cumulative receiver concentration of QC compounds, lucifer yellow, atenolol, and caffeine, at appropriate time points, and their P_{app}, and percent recovery on the parallel control assay are shown in Table 2. The raw data for the cumulative concentration of the Customer's test compound, danazol, in the receiver side at appropriate time points, and the fluxes from formulation are shown in Table 3.

Tissue integrity marker, lucifer yellow, was below lower limit of quantification (LLOQ) in all receiver buffer. The P_{app} of low permeable control compound, atenolol, exhibited $0.02 \pm 0.04 \times 10^{-6}$ cm/s and that of high permeable control compound, caffeine, exhibited $0.10 \pm 0.12 \times 10^{-6}$ cm/s. The flux of the customer's test compound, danazol, was zero in all replicate, since danazol was not detected in all receiver compartment.

Table 2. The Raw Data of Donor and Cumulative Receiver Concentration (μM), P_{app}, and Percent Recovery of Control Compounds, Lucifer Yellow, Atenolol, and Caffeine, on In-Parallel Control Assay

Test Compound	Sampling Side	Sampling Time	Replicate 1	Replicate 2	Replicate 3	Mean ± S.D.
Lucifer Yellow	Donor	Dosing	111.9	100.4	99.9	104.0 ± 6.8
		0 hors	100.4	99.9	97.5	99.3 ± 1.6
		48 hours	86.6	95.1	84.5	88.7 ± 5.6
	Receiver	0 hours	0*	0*	0*	0 ± 0
		2 hours	0*	0*	0*	0 ± 0
		4 hours	0*	0*	0*	0 ± 0
		8 hours	0*	0*	0*	0 ± 0
		24 hours	0*	0*	0*	0 ± 0
		48 hours	0*	0*	0*	0 ± 0
	Papp ($\times 10^{-6}$cm/s)		0	0	0	0 ± 0
	% Recovery		83.2	91.4	81.2	85.3 ± 5.4
Atenolol	Donor	Dosing	102.0	101.0	103.0	102.0 ± 1.0
		0 hors	93.6	91.9	94.7	93.4 ± 1.4
		48 hours	96.2	96.2	83.1	91.8 ± 7.6
	Receiver	0 hours	0*	0*	0*	0 ± 0
		2 hours	0*	0*	0.003	0.001 ± 0.002
		4 hours	0*	0*	0.007	0.002 ± 0.004
		8 hours	0*	0*	0.022	0.007 ± 0.013
		24 hours	0.001	0*	0.110	0.037 ± 0.063
		48 hours	0.003	0*	0.212	0.072 ± 0.122
	Papp ($\times 10^{-6}$cm/s)		0.00	0	0.06	0.02 ± 0.04
	% Recovery		94.3	94.3	82.3	90.3 ± 6.9
Caffeine	Donor	Dosing	110.0	107.0	109.0	108.7 ± 1.5
		0 hors	103.0	102.0	103.0	102.7 ± 0.6
		48 hours	112.0	105.0	90.1	102.4 ± 11.2
	Receiver	0 hours	0*	0*	0*	0 ± 0
		2 hours	0*	0*	0.026	0.009 ± 0.015
		4 hours	0.001	0.001	0.053	0.018 ± 0.030
		8 hours	0.007	0.007	0.137	0.050 ± 0.075
		24 hours	0.055	0.044	0.489	0.196 ± 0.254
		48 hours	0.132	0.106	0.931	0.390 ± 0.469
	Papp ($\times 10^{-6}$cm/s)		0.04	0.03	0.24	0.10 ± 0.12
	% Recovery		103.6	97.0	86.3	95.6 ± 8.7

* ; Below the lower limit of quantification. Assumed as 0 for calculation of P_{app} and % recovery.

Table 3. The Raw Data of Cumulative Concentration (μM) in the Receiver Side and Flux of Test Compound, Danazol, from Formulation

Sampling Side	Sampling Time	Replicate 1	Replicate 2	Replicate 3	Mean ± S.D.
Receiver	0 hours	0*	0*	0*	0 ± 0
	2 hours	0*	0*	0*	0 ± 0
	4 hours	0*	0*	0*	0 ± 0
	8 hours	0*	0*	0*	0 ± 0
	24 hours	0*	0*	0*	0 ± 0
	48 hours	0*	0*	0*	0 ± 0
Flux (nmole/cm^2/hrs)		0	0	0	0 ± 0

* ; Below the lower limit of quantification. Assumed as 0 for calculation of flux.

4. DISCUSSION AND CONCLUSIONS

Tissue integrity marker, lucifer yellow, was below LLOQ in all receiver buffer. The acceptance criteria of the lucifer yellow P_{app} for preserved dermatomed skin tissue integrity is $0 - 0.2 \times 10^{-6}$ cm/s. The results indicate that the donor used in this assay was acceptable until 48 hours on the tissue integrity assessment, since all replicate was passed by lucifer yellow permeation. The P_{app} of low permeable control compound, atenolol, exhibited $0.02 \pm 0.04 \times 10^{-6}$ cm/s and that of high permeable control compound, caffeine, exhibited $0.10 \pm 0.12 \times 10^{-6}$ cm/s.

The flux of the customer's test compound, danazol, was zero in all replicate, since danazol was not detected in all receiver compartment. A probable cause was the customer's formulation, a solid cream type formulation, which was not suitable for the Franz-cell diffusion assay utilized in this assay because of the difficulties of spreading on the skin surface to cover the entire surface tightly by the characteristics of this formulation. Another possibility is that the danazol may also have accumulated in the skin tissue and not have been released into the receiver compartment since danazol is very insoluble and lipophilic.

APPENDIX I
Analytical Methodology

Liquid Chromatography

Column:	Keystone Hypersil BDS C18 30x2.0 mm i.d., 3 µm, with guard column
M.P. Buffer:	25 mM ammonium formate buffer, pH 3.5
Aqueous Reservoir (A):	90% water, 10% buffer
Organic Reservoir (B):	90% acetonitrile, 10% buffer
Flow Rate:	300 µL/minute
Gradient Program:	

Time (Min)	% A	% B
0.0	100	0
1.5	0	100
2.0	0	100
2.1	100	0
3.5	100	0

Total Run Time:	3.5 minutes
Autosampler:	30 µL Injection Volume
Autosampler Wash:	water/acetonitrile/2-propanol:1/1/1; with 0.2% formic acid

Mass Spectrometer

Instrument:	PE SCIEX API 3000
Interface:	Turbo Ionspray
Mode:	Multiple Reaction Monitoring
Method:	3.5 minute duration
Settings:	

Compound	Q1/Q3	DP	EP	CP	CE	CXP	IS	TEM	NEB	CUR	CAD
Danazol	+338.2/148.3	85	200	12	34	8	5500	500	8	8	12
Atenolol	+267.2/145.1	40	200	10	30	5	5500	500	8	8	7
Caffeine	+195.0/138.1	60	200	10	30	5	5500	500	8	8	7

STUDY REPORT NO.:

02-FEMM.P01.R1 ~ Report 1
REVISION 1

STUDY REPORT TITLE:

Danazol Permeation of Dermatomed Human Breast Skin

PREPARED FOR:
FEMME PHARMA, INC.

DATE OF ISSUE: JANUARY 31, 2003
REVISION 1 ISSUED: FEBRUARY 6, 2003

CONFIDENTIAL

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OBJECTIVE

The objective of this study was to assess the skin permeability characteristics of danazol using dermatome human female breast skin. Two vehicles were prepared and evaluated: propylene glycol and 5% oleyl alcohol in propylene glycol. In addition, lucifer yellow was included in each dosing vehicle as a marker compound to assess membrane barrier integrity of each membrane during the course of the experiment. A copy of the study protocol derived from the agreed to scope of work is included as Appendix 1 of this report.

EXPERIMENTAL

Materials

Lucifer yellow was purchased from Molecular Probes (Eugene, OR). Bovine serum albumin, oleyl alcohol and propylene glycol were obtained from Sigma-Aldrich (St.Louis, MO). The reservoir buffer consisted of filtered 1% bovine serum albumin (BSA) in Krebs Ringer bicarbonate (KRB) buffer containing 10 mM HEPES and 0.015 mM sodium bicarbonate at the pH of 7.4. Femme Pharma supplied danazol.

Tissue

Dermatomed human breast skin was obtained from Bioreclamation Inc. (Hicksville, NY). The donor was a 72 year old, Caucasian female. The dermatomed skin consists only of epidermal layer and was kept frozen at -80°C until the time of the study.

Dosing Vehicles

Danazol solubility in propylene glycol was >10 mg/mL. Two dosing vehicles were prepared containing 10 mg/mL danazol (1% w/v). The vehicles were propylene glycol and 5% oleyl alcohol in propylene glycol. Oleyl alcohol is known to have skin permeation enhancing properties. In addition, these vehicles contained 2.5 mg/mL lucifer yellow. Lucifer yellow was added to monitor membrane integrity during the experiment. Each dosing vehicle was run in four replicates from the one skin donor. The dosing vehicles are described in Table 1.

Table 1. Composition of Dosing Vehicles

Propylene Glycol			
Compound	**Concentration**	**Catalog**	**lot**
10 mL propylene glycol		Sigma P-4347	111K1658
25.63 mg lucifer yellow	2.5 mg LY / mL	Mol. Probes L-453	28B1-3
100.64 mg danazol	10 mg / mL	Femme Pharma	20020902
5% Oleyl Alcohol in PG			
Compound	**Concentration**	**Catalog**	**lot**
9.5 mL propylene glycol	95%	Sigma P-4347	111K1658
0.5 mL oleyl alcohol	5%	Aldrich 369314	20321KA
25.46 mg lucifer yellow	2.5 mg LY / mL	Mol. Probes L-453	28B1-3
100.61 mg danazol	10 mg / mL	Femme Pharma	20020902

Permeation Study

The skin was thawed at room temperature for approximately 30 minutes and rinsed with saline. The skin was cut into approximately 3 cm^2 sections and sections were clamped between the donor and receiver chambers of Franz diffusion cells. The receptor compartment was filled with 8 mL of reservoir buffer and contained a stirring bar to mix the reservoir contents. The dosing vehicle (0.2 mL) was then placed directly on top of the skin in the donor compartment. Each Franz diffusion cell was placed in a dry block heating/stirring module. The temperature was set at 40°C in order to maintain 37° C in the reservoir. The stirring rate was set at 10 (400 RPM). Samples (0.5 mL) were taken from the reservoir compartment at 2, 4, 8, 24, 32, and 48 hours and replaced with an equal volume of reservoir buffer. For the analysis of danazol, 200 µL of reservoir sample was diluted with 400 µL acetonitrile to precipitate the albumin, and centrifuged at 10,000 RPM for 10 minutes. At the end of the 48 hours incubation, samples were collected from the donor compartment for evaluating mass balance.

Sample Analyses

Lucifer yellow concentrations were measured using a FLUOstar fluorescence plate reader (BMG Laboratories, Durham, NC). The excitation and emission wavelengths were 485 and 538 nm, respectively. Danazol was measured by LC/MS using electrospray ionization. The method is described in Appendix 2.

Data Analysis

Cumulative concentrations in the receiver chamber were calculated compensating for the removal and replacement of the 0.5 mL sample, as follows.

$$C_r = C^n + (0.5\ mL / 8.0\ mL) \times C^{n-1}$$

where C_r and C^{n-1} are the measured receiver concentrations at time point n, and the previous time point, n-1, respectively.

The apparent permeability, Papp, was calculated as follows:

$$Flux = (dC_r/dt) \times V_r / A$$
$$Papp = (dC_r/dt) \times V_r / (A \times C_0)$$

where,
dC_r/dt is the slope cumulative concentration in the receptor compartment versus time in µg/mL
V_r is the volume of the receptor compartment in 8 mL
V_d is the volume of the donor compartment in 0.20 mL
A is the diffusional area of the exposed skin membrane, 1.77cm^2
C_0 is the initial concentration of compound in the dosing vehicle in µg/mL.

RESULTS

Danazol Permeation

A plot of the amounts of danazol permeating through the breast skin vs. time is given in Figure 1. Skin permeability of danazol was clearly enhanced in the presence of 5% oleyl alcohol. Flux and Papp were estimated using the slope of the cumulative concentration vs. time profiles from 8 hours to 48 hours. Flux and Papp values are presented in Table 2.

Figure 1. Cumulative skin permeation of danazol using propylene glycol and 5% oleyl alcohol in propylene glycol vehicles.

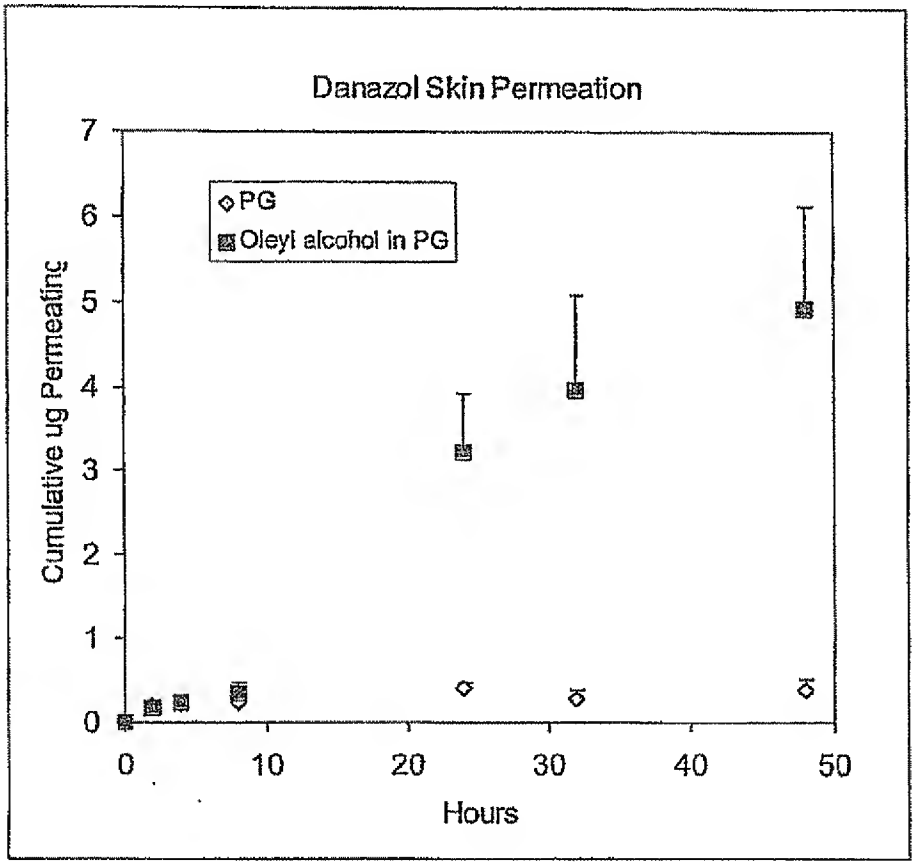

Table 2. Danazol flux and Papp values.

Dosing Vehicle	Danazol Flux (ug/cm^2/hr)	Danazol Papp (10^{-6}, cm/hr)
Propylene glycol (PG)	0.0034 ±0.0015	0.32 ± 0.14
5% Oleyl alcohol in PG	0.055 ± 0.016	4.83 ± 0.40

The donor chambers were sampled at the end of the 48 hour incubation period and assayed for danazol. These results are given in Table 3. The propylene glycol vehicle provided relatively low permeation as indicated by high percentages recovered in the donor compartment, consistent with the results for permeation through the skin. After dosing with the oleyl alcohol / PG vehicle only low percentages remained in the donor compartment at 48 hours, indicating extensive permeation into and/or through the skin.

Table 3. Danazol donor concentrations after the 48 hour incubation, and percentage remaining unabsorbed.

Danazol in Propylene Glycol					
Time (Hr.)	Skin 1	Skin 2	Skin 3	Skin 4	Ave (1-3 only)
0	Not individually sampled				10.90
48	12.00	9.37	8.73	3.47	
Remaining %	110.09	85.96	80.09	31.83	92.05
Danazol in 5% Oleyl Alcohol / 95% Propylene Glycol					
Time (Hr.)	Skin 5	Skin 6	Skin 7	Skin 8	Ave
0	Not individually sampled				13.30
48	1.43	1.50	1.14	1.01	
Remaining %	10.75	11.28	8.57	7.59	9.55

Lucifer Yellow Permeation

Each skin membrane was evaluated for permeation of lucifer yellow, which provides an indication of membrane integrity. There was no permeation of lucifer yellow detectable until after 8 or 24 hours of incubation, indicating that these skin specimens were not leaky to this polar marker compound. Lucifer yellow Papp values were similar for the propylene glycol and oleyl alcohol vehicles.

CONCLUSIONS

The permeability of danazol was approximately 13-fold greater using the vehicle comprised of 5% oleyl alcohol in propylene glycol, relative to propylene glycol. The extensive disappearance of danazol from the donor with the oleyl alcohol / PG vehicle suggests that danazol could also be permeating into the skin and remaining there, not having great affinity for the albumin in buffer reservoir.

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Appendix 1. Study Protocol

OBJECTIVE

To assess the permeability of Danazol through human skin

Receipt of sample

A. In powder form - ≥ amount 5 g

Analytical Methods Suitability

A. MS Optimization
B. LC Optimization
C. Specificity, Sensitivity, Accuracy

Solubility Assessment

A. Assessment of test article solubility

Tissue Qualification

A. Incorporation of Lucifer Yellow in vehicle as a tissue integrity marker

Permeability Assessment in Human Skin (Franz Diffusion Cell)

A. Human skin will be obtained and the following studies will be run:
    1. Evaluation of test article Danazol
        a. Two (2) vehicles
            1. Propylene Glycol (PG)
            2. Oleyl alcohol (5%) in PG
        b. One (1) concentration of Danazol (TBD based on solubility)
        c. Uni-directional permeability
        d. N=3 replicates per vehicle
        e. Sampling time points
            1. Sample receiver at six (6) time points: 2,4, 8, 24, 32, and 48 hours
    2. Lucifer Yellow control

Report Generation

A. Data Processing and Interpretation
B. Issue Report
C. Flux measurement for Danazol in two different vehicles

Appendix 2. Danazol analytical method

Liquid Chromatography

Column: Keystone Hypersil BDS C18 30x2.0 mm i.d., 3 µm, with guard column

M.P. Buffer: Ammonium Formate Buffer, pH 3.5
Aqueous Reservoir (A): 90% water, 10% buffer
Organic Reservoir (B): 90% acetonitrile, 10% buffer

Flow Rate: 300 µL/min.

Gradient Program (typically):

Time	Grad. Curve	% A	% B	TE3	TE4
-0.1	0	50	50	close	
1.2	1	30	70		close
3.0	1	0	100		
3.1	0	50	50		
4	0	50	50	close	

Total run time: 4.5 min

Autosampler: 10 µL Inj. Vol.

Autosampler wash: water/acetonitrile/2-propanol::1/1/1; with 0.2% formic acid

Mass Spectrometer

(Typical Operating Conditions)

Interface: APCI

Mode: Single Ion Monitoring (Positive mode, m/z = 338.2)

Gases: Neb Gas = 8, Curtain Gas = 10, Turbo Ionspray Gas = 8000 mL/min.

TEM: 350°C

Voltages: IS 4500, OR 25, RNG 200, Q0 –10, IQ1 –12, ST –15, RQ0 –12, DF –200, CEM (per age)

Method: 4.5 minute duration.

S L Pharma Labs

Feb 12, 2004

Formulation Development Summary on Danazol

Progress Report Number 4

Five topical formulations of danazol were prepared at target concentrations of 2 to 5% w/w. Placebo preparations were also made for each formulation. The formulations include:

1. Hydro-alcoholic gels: prepared using alcohol and other water miscible organic solvents
2. Non-aqueous gel: prepared using water miscible organic solvents
3. Polyethylene glycol (PEG) ointments: water soluble ointment base

1. Hydro-alcoholic gels:

Two hydro-alcoholic gel formulations were prepared at 2% w/w danazol concentration. These gels were prepared using the gelling agent, Carbomer 940. No precipitation of danazol was observed on room temperature storage. First gel formulation contains only about 6% dehydrated alcohol which is used primarily to disperse the gelling agent and also to provide some cooling effect.

Hydro-alcoholic Gel # 1

No.	Ingredient	% w/w
1	Danazol	2.0
2	Water	10.0
3	PEG 400	24.0
4	2-Pyrrolidone	47.1
5	Propylene glycol	5.0
6	Glycerin	5.1
7	Dehydrated alcohol	6.0
8	Carbomer 940	0.8
Light yellow, clear, viscous, fluid, smooth, and non-stringy gel		
pH: 6.77		

FPPD-1 p. 176, 183

The second formulation contains about 47% alcohol which is used as a co-solvent to solubilize danazol and to provide a significant cooling effect.

S L Pharma Labs

Feb 12, 2004

Hydro-alcoholic Gel # 2

No.	Ingredient	% w/w
1	Danazol	2.0
2	Water	10.0
3	PEG 400	15.0
4	2-Pyrrolidone	15.0
5	Dehydrated alcohol	46.9
6	Propylene glycol	5.0
7	Glycerin	5.0
8	Carbomer 940	1.0
9	1% Aqueous soln. of Trometharnine	To adjust pH
Light yellow, clear, thick, smooth, and non-stringy gel.		
pH: 6.63		

FPPD-1 p. 178, 183

2. Non-aqueous gel prepared using water miscible organic solvents:

The non-aqueous gel formulation containing 4% w/w Danazol was prepared using the gelling agent, hydroxypropyl cellulose (Klucel). The non-aqueous gel formulations presented in Progress Report # 3 were further modified to include higher percentage of dehydrated alcohol. The modification was done to obtain a smooth and non-stringy gel formulation to improve the feel of gel on topical application. The use of higher alcohol percentage in the formulation also provides a significant cooling effect. No precipitation of danazol was observed on room temperature storage.

Non-aqueous Gel

No.	Ingredient	% w/w
1	Danazol	4.0
2	PEG 400	39.9
3	2-Pyrrolidone	10.0
4	Propylene glycol	8.0
5	Dehydrated alcohol	26.8
6	Glycerin	10.1
7	Klucel	1.25
Light yellow, clear, smooth, and non-stringy gel		
pH: 7.12		

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3. PEG ointments:

Two PEG ointment formulations were prepared using different concentrations of PEG 400 and PEG 3350 based on the method outlined in USP. The two formulations were prepared at 3% and 5% w/w Danazol concentrations, respectively. No danazol crystals were observed through microscopic examination on room temperature storage. The two formulations are presented below:

PEG Ointment #1: 3% w/w Danazol

No.	Ingredient	% w/w
1	Danazol	3.1
2	PEG 400	51.9
3	PEG 3350	20.0
4	2-Pyrrolidone	15.1
5	Glycerin	10.0
Light yellow, opaque ointment		

FPPD-1 p. 166-167

PEG Ointment #2: 5% Danazol

No.	Ingredient	% w/w
1	Danazol	5.0
2	PEG 400	45.0
3	PEG 3350	34.9
4	2-Pyrrolidone	10.0
5	Glycerin	5.0
Light yellow, opaque, very firm ointment		

FPPD-1 p. 181

Conclusion and Recommendations:

All of these formulations contain danazol at the desired target concentration range of 2 to 5% w/w. These formulations are observed to have a good feel upon topical skin application.

These formulations need further evaluation to determine the extent of skin penetration of danazol and skin sensitivity. Based on the results of these two studies, the formulations can be modified in future to adjust the danazol concentration and the concentration of other excipients.

In addition, these formulations should be evaluated for preservative effectiveness along with accelerated and long term stability.

STUDY REPORT NO.:

04-FEMM.P01R2 ~ REPORT 1

FINAL REPORT

STUDY REPORT TITLE:

Danazol Permeation Through Dermatomed Human Breast Skin

PREPARED FOR:
FEMME PHARMA, INC.

DATE OF ISSUE: MAY 21, 2004

CONFIDENTIAL

Prepared by:

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OBJECTIVE

The objective of this study was to assess the skin permeability characteristics of danazol in three dosing vehicles using dermatomed human female breast skin. In addition, lucifer yellow, caffeine and atenolol were included in each dosing vehicle. Lucifer yellow was used as a marker compound to assess membrane barrier integrity of each skin specimen during the course of the experiment. Caffeine and atenolol served as reference compounds with high and moderate permeability potential, respectively.

EXPERIMENTAL

Materials
Lucifer yellow was purchased from Molecular Probes (Eugene, OR). Bovine serum albumin (BSA), Krebs Ringer bicarbonate buffer, atenolol, and antipyrine were obtained from Sigma-Aldrich (St.Louis, MO). The reservoir buffer consisted of filtered 1% BSA in Krebs Ringer bicarbonate (KRB) buffer containing 10 mM HEPES and 0.015 mM sodium bicarbonate at a pH of 7.4. Femme Pharma supplied the three formulated danazol gels.

Tissue
Dermatomed human breast skin was obtained from Bioreclamation Inc. (Hicksville, NY). The donor was a 72 year old, Caucasian female. The dermatomed skin consists only of epidermal layer and was kept frozen at -80°C until the time of the study.

Dosing Vehicles
Femme Pharma supplied the three formulated danazol gels. The danazol gels were Hydro-alcoholic gel #1 (active), Hydro-alcoholic gel #2 , and PEG ointment #1. To these vehicles, prior to dosing, lucifer yellow, caffeine and atenolol were added at 2.5 mg/mL. Each dosing vehicle was run in three replicates.

Non-specific Binding Studies
Non-specific binding to the Franz chamber apparatus was assessed by exposing the danazol to the apparatus without tissue (blank apparatus). The concentration of danazol was 10 µg/mL. The concentration in the apparatus was determined after 0 and 180 minutes of incubation to mimic a skin permeation experiment. The medium was filtered 1% bovine serum albumin (BSA) in Krebs Ringer bicarbonate (KRB) buffer containing 10 mM HEPES and 0.015 mM sodium bicarbonate at a pH of 7.4. The experiment was done in triplicate. The danazol concentrations in the buffer did not change significantly during the course of the experiment, indicating that there was no significant loss to the apparatus as outlined below:

Time (min)	Average Measured Concentration (µg/mL) ± STD (N=3)
0	8.9 ± 0.3
180	8.5 ± 0.3

Permeation Study
The skin was thawed at room temperature for approximately 30 minutes and rinsed with saline. The skin was cut into approximately 3 cm^2 sections, and the sections were clamped between the donor and receiver chambers of Franz diffusion cells. The receiver compartment was filled with 8 mL of reservoir buffer and

contained a stirring bar to mix the reservoir contents. The dosing gel vehicle (0.2 mL) was then placed directly on top of the skin in the donor compartment. Each Franz diffusion cell was placed in a dry block heating/stirring module. The temperature was set at 40°C in order to maintain 37° C in the reservoir. The stirring rate was set at 10 (400 RPM). Samples (1 mL) were taken from the receiver compartment at 0, 2, 4, 8, 24, 32, and 48 hours and replaced with an equal volume of reservoir buffer. For the analysis of danazol, 100 µL aliquots of the receiver samples were placed in a 2-mL conical bottom 96-well plate, and 400 µL of acetonitrile was added to each sample. The plate was centrifuged at 3500 rpm for 15 minutes to precipitate the protein in the receiver samples. The 400 µL of supernatant was transferred to a new plate and the organic was evaporated at 45 °C for 30 min before reconstitution with 100 µL of 10 % acetonitrile in deionized water. The samples were vortexed for 5 minutes and 15 µL was injected on to the LC/MS. At the end of the 48 hours of incubation, all gel was collected from the donor and analyzed.

Sample Analysis
Lucifer yellow concentrations were measured using a FLUOstar fluorescence plate reader (BMG Laboratories, Durham, NC). The excitation and emission wavelengths were 485 and 538 nm, respectively. Danazol was measured by LC/MS using electrospray ionization. The method is described in Appendix 1.

Data Analysis
Cumulative concentrations in the receiver chamber were calculated compensating for the removal and replacement of the 1.0 mL sample, as follows.

$$C_r = C^n + (1\ mL / 8.0\ mL) \times C^{n-1}$$

where,
C_r is the cumulative concentration in the receiver compartment
C^n is the measured receiver concentration at time point n
C^{n-1} is the measured receiver concentration at the previous time point, n-1

The apparent permeability, Papp, and Flux were calculated as follows:

$$Flux = (dC_r/dt) \times V_r / A$$
$$Papp = (dC_r/dt) \times V_r / (A \times C_0)$$

where,
dC_r/dt is the slope of the cumulative concentration in the receiver compartment versus time
V_r is the volume of the receiver compartment, 8 mL
V_d is the volume of the donor compartment, 0.20 mL
A is the diffusional area of the exposed skin membrane, 1.78cm^2
C_0 is the initial concentration of compound in the dosing vehicle in µg/mL

For the test article danazol and other assayed compounds, the linear portion of the cumulative concentration in the receiver compartment versus time was used to calculate the Papp and Flux value.

RESULTS

Danazol Permeation

A plot of the amount of danazol permeating through the breast skin vs. time is given in Figure 1. Papp and Flux values of danazol are presented in Tables 1 and 2. Lucifer yellow, caffeine and atenolol Papp values are presented in Table 3. The donor chambers were sampled at the end of the 48-hour incubation period and assayed for danazol. These results are given in Table 4.

Figure 1. Cumulative Skin Permeation of Danazol in Three Tested Vehicles

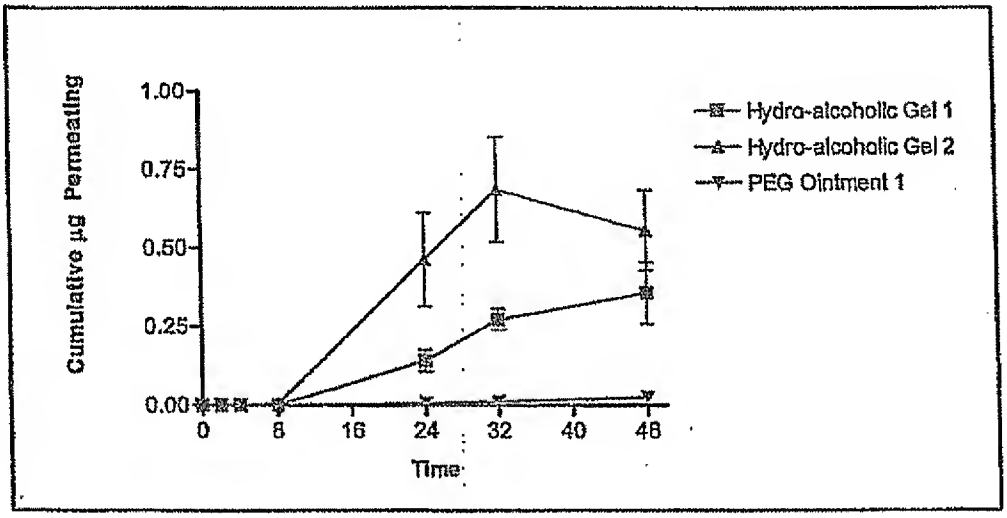

Table 1. Danazol Papp Values

Dosing Vehicle	Papp (10^{-5}, cm/hr) Replicate 1	Papp (10^{-6}, cm/hr) Replicate 2	Papp (10^{-6}, cm/hr) Replicate 3	Average Papp (10^{-6}, cm/hr) ± STD
Hydro-alcoholic gel #1	1.153	2.418	1.566	1.712 ± 0.645
Hydro-alcoholic gel #2	2.752	6.335	3.537	4.208 ± 1.884
PEG Ointment #1	0.131	0.177	0.087	0.131 ± 0.045

Table 2. Danazol Flux Values

Dosing Vehicle	Flux ($\mu g/cm^2/hr$) Replicate 1	Flux ($\mu g/cm^2/hr$) Replicate 2	Flux ($\mu g/cm^2/hr$) Replicate 3	Average Flux ($\mu g/cm^2/hr$) ± STD
Hydro-alcoholic gel #1	0.027	0.062	0.036	0.041 ± 0.018
Hydro-alcoholic gel #2	0.083	0.189	0.111	0.127 ± 0.055
PEG Ointment #1	0.004	0.005	0.002	0.004 ± 0.001

Table 3. Papp Values of Lucifer Yellow, Atenolol and Caffeine

Dosing Vehicle	Average Lucifer Yellow Papp (10^{-6}, cm/s) ± STD (N=3)	Average Atenolol Papp (10^{-6}, cm/s) ± STD (N=3)	Average Caffeine Papp (10^{-6}, cm/s) ± STD (N=3)
Hydro-alcoholic Gel #1	0.0020 ± 0.0026	0.0016 ± 0.0024	0.0498 ± 0.0098
Hydro-alcoholic Gel #2	0.0010 ± 0.0004	0.0014 ± 0.0014	0.1188 ± 0.0717
PEG Ointment #1	NC*	0.00005 ± 0.00003	0.0108 ± 0.0009

* *Values were below lowest limit of quantification. Therefore, Papp was not calculated.*

The donor chambers were sampled at the end of the 48-hour incubation period and assayed for danazol. These results are given in Table 4. The propylene glycol vehicle provided relatively low permeation as indicated by high percentages recovered in the donor compartment, which is consistent with the results for permeation through the skin. After dosing with the oleyl alcohol / PG vehicle, only low percentages remained in the donor compartment at 48 hours, indicating extensive permeation into and/or through the skin.

Table 4. Danazol Donor Concentrations After the 48-hour Incubation and Percentage Remaining Unabsorbed

Hydro-alcoholic Gel #1 (µg/mL, 10^3)				
Time (Hr.)	Replicate 1	Replicate 2	Replicate 3	Average
0	23.1	25.5	22.7	23.8
48	2.46	6.96	14.3	7.91
% Remaining	10.6	27.3	63	33
Hydro-alcoholic Gel #2 (µg/mL, 10^3)				
Time (Hr.)	Replicate 1	Replicate 2	Replicate 3	Average
0	30.1	29.8	31.3	30.4
48	6.84	3.40	2.14	4.13
% Remaining	22.7	11.4	6.8	14
PEG Ointment #1 (µg /mL, 10^3)				
Time (Hr.)	Replicate 1	Replicate 2	Replicate 3	Average
0	29.4	29.5	28.0	29.0
48	20.4	11.5	15.0	15.6
% Remaining	69.4	39.0	53.6	54

COMMENTS AND CONCLUSIONS

Caffeine and atenolol represent highly absorbed and moderately absorbed reference compounds, respectively. For each tested tissue, the reference compounds had Papp values with the expected rank order of caffeine > atenolol. In addition, Papp values of lucifer yellow were comparable to Papp values of atenolol. Also, there was no permeation of lucifer yellow detectable until after 8 or 24 hours of incubation, which attests to the integrity of the tissue.

The permeability of danazol was higher when applied in Hydro-alcoholic gels compared to PEG ointment. The Papp and Flux rank order of danazol was: Hydro-alcoholic gel # 2> Hydro-alcoholic gel # 1 > PEG ointment.

SIGNATURES

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Appendix 1. Danazol Analytical Method

LC/MS Conditions

Liquid Chromatography

Column: Hypersil BDS C18 30 x 2.1 mm i.d., 3µ, Thermo Electron Corporation

Aqueous M.P. (A): Water
Organic M.P. (B): ACN

Flow Rate: 300 µL/min

Gradient Program (typically):

Time (min)	Grad. Curve	% A	% B	Waste	MS
0.0	0	95	5		
1.0	1	64	36		close
3.0	1	0	100		
4.5	0	0	100	close	
4.6	1	95	5		
5.5	0	95	5		

Total run time: 6 min

Autosampler: 15 µL Inj. Vol.

Autosampler wash: water/acetonitrile/2-propanol: 1/1/1; with 0.2% formic acid

Mass Spectrometer

(Typical Operating Conditions)

Mass Spectrometer APCI 3000, Triple Quadrupole LC/MS/MS

Interface: Heated Nebulizer

Mode: Multiple Reaction Monitoring

Gases: NEB = 15, Curtain Gas = 6

TEM: 550°C

Voltages: IS 5000, DP 46, FP 170, EP 10

Method: 6.0 minute duration